# Center for Veterinary Biologics and National Veterinary Services Laboratories Testing Protocol

## Supplemental Assay Method for the Determination of Protein and Phenol in PPD (Purified Protein Derivative Produced From Cultures of *Mycobacterium bovis* Strain AN-5) Bovis Tuberculin

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## 1. Introduction

The Code of Federal Regulations, Title 9 (9 CFR) (Animals and Animal Products) states that the Animal and Plant Health Inspection Service(APHIS) is responsible for administering the Virus-Serum-Toxin Act. It requires licensed tuberculin be tested and approved by the Center for Veterinary Biologics (CVB) before it can be marketed. Protein concentration is determined by classical Kjeldhal digestion, distillation, and titration of the ammonia. Phenol is determined by end-point titration with bromate/bromide. Satisfactory product must contain 1.0 mg/ml ± 0.1 mg/ml protein. Phenol content must be 0.50% ± 0.04%.

## 2. Materials

### 2.1 Equipment

**2.1.1** Balance, top loading, capable of measuring 0.01 g

**2.1.2** Digestion unit, Buchi, B-426, with digestion tubes

**2.1.3** Distillation unit, Buchi, B-316

**2.1.4** Volumetric pipets, Class A, meets ASTM Standard E969-83

**2.1.5** Volumetric flasks, Class A, with barrel head glass stopper, meets ASTM E288 requirements

**2.1.6** 125-ml erlenmeyer flasks

**2.1.7** 10-ml buret with PTFE stopcock, precision bore, calibrated to ASTM E-694 accuracy requirements

**2.1.8** 50-ml buret with PTFE stopcock, precision bore, calibrated to ASTM E-694 requirements

**2.1.9** Graduated cylinders, 50, 100, 250, 500, and 1,000 ml, (PYREX), meets ASTM D86, D216, D447 requirements

**2.1.10** Glass-stoppered erlenmeyer flasks, 250 ml

**2.1.11** Heating/stirring plate with stirring bars

**2.1.12** Fast filter paper

**2.2 Reagents/Supplies:** All chemicals, reagent grade. Use distilled or demineralized water or water of equivalent purity.

**2.2.1 Protein**

**1.** Sulfuric acid ($H_2SO_4$)--Purity: Minimum 95.0%, Maximum 98.0%

**2.** Mercury Tablets, Brinkmann Instruments, Catalog No. 015-00-646-3

**3.** Sodium hydroxide (NaOH)--Purity: 98.5%

**4.** Boric acid ($H_3BO_3$)--Purity: 99.9%

**5.** Methyl red--Purity: 98.0%

**6.** Hydrochloric acid (HCl)--Assay: 36.5%-38.0%

**7.** Sodium carbonate ($Na_2CO_3$)--Purity: 99.9%

**8.** Bromo phenol blue--Purity: 98.0%

**9.** National Veterinary Services Laboratories (NVSL) Control--Pool of PPD Tuberculin products with established protein and phenol values

**10.** Protein, Bovuminar crystallized powder--Intergen Company, 2 Manhattanville Road, Purchase, NY 10577, Catalog No. 3000-70, Purity: 98.9%

**2.2.2 Phenol** (some reagents same as for protein)

**1.** Methyl orange--Purity: 98.0%

**2.** Silicotungstic acid ($H_4[Si(W_3O_{10})_4]*26H_2O$)--Purity: 99.0%

**3.** Arsenic trioxide ($As_2O_3$)--Purity: 99.9%

**4.** Sodium bicarbonate ($NaHCO_3$)--Purity: 99.9%

**5.** Potassium bromate ($KBrO_3$)--Purity: 98.5%

**6.** Potassium bromide (KBr)--Purity: 99.0%

**7.** Phenol ($C_6H_5OH$)--Purity: $\geq$ 99.0%

## 3. Preparation for the Test

### 3.1 Training of technical personnel

No special test-related training is needed for this testing. Analysts performing this procedure should first conduct 2 trial runs using controls and standards and obtain results within acceptable limits.

### 3.2 Preparation of equipment/instrumentation

Become familiar with Buchi instruction regarding operation. Turn on water that aspirates fume from suction tube of the digestion unit and keep the water cool in the condenser of the distillation unit. Adjust water flow in the distillation unit to approximately 1 L per min. Turn on the distillation unit. Set time preselector to “2” (2 min) and stopcock for aspiration to “Off.” Make sure that Buchi bottles of NaOH and water are adequate.

### 3.3 Preparation of reagents

**3.3.1 Protein Test** (all reagents stable for at least 6 mo unless specified)

**1.** Cut Hg tablets into half.

**Caution: Because tablet contains mercury, handle in fume hood and wear gloves, protective glasses, and mask.**

**2.** 32% NaOH, dissolve 640 g ± 1 g NaOH in 1.4 L $H_2O$ in 2-L volumetric flask on the magnetic stirrer. Cool to room temperature. Dilute to volume with $H_2O$. Repeat above until Buchi 10-L bottle is full. Store at 4°C.

**Caution: NaOH is caustic--Avoid contact with skin.**

**3.** Saturated $H_3BO_3$, add 15 g to 100 ml $H_2O$. Stir, with heat, until all $H_3BO_3$ dissolves. Some $H_3BO_3$ recrystallizes when cool. Store at room temperature (RT).

**4.** 0.5% methyl red, dissolve 0.5 g in 100 ml ethanol. Store at RT. Stable for 2 mo.

**5.** Standardized 0.01 N HCl-0.02 N HCl, 1.7 ml HCl/L $H_2O$. Titrate exactly 0.0100 g dried sodium carbonate dissolved in 25 ml $H_2O$. Indicator: 3 drops 0.1% bromo phenol blue; the color of endpoint is green not bluish green, nor yellowish green. Store at RT.

**Calculation:**

$$\underline{N}\ HCl = [(g\ Na_2CO_3)x(1000)]/[(Vol\ HCl)x(52.994)].$$

**Caution: Concentrated HCl is corrosive--Handle in fume hood. Avoid contact with skin.**

**6.** Protein standard, weigh approximately 2 g Bovuminar crystallized powder and transfer to 2-L flask. Dissolve and dilute to 2 L with $H_2O$. Aliquot into 15-ml portions in 30-ml serum vials. Seal under nitrogen. Store at 4°C.

**3.3.2 Phenol Test** (all reagents stable for at least 6 mo unless specified)

**1.** 20% HCl, slowly add 200 ml HCl to 600 ml $H_2O$, dilute to 1 L. Store at RT.

**2.** 0.1% methyl orange, add 0.1 g methyl orange to 100 ml $H_2O$. Filter if necessary. Make fresh every 2 mo. Store at RT.

**3.** Silicotungstic acid solution (SAS), dissolve 60 g $H_4[Si(W_3O_{10})_4]*26H_2O$ in 400 ml $H_2O$ in 500-ml volumetric flask. Add 50 ml $H_2SO_4$. When cool, dilute to volume with $H_2O$. Store at 4°C.

**4.** Clarifying solution (CS), add 50 ml SAS and 125 ml 20% HCl to 325 ml $H_2O$. Prepare fresh prior to each test.

**5.** "Acid solution" for $As_2O_3$ standard solution, add 110 ml HCl and 2.5 ml methyl orange to 100 ml $H_2O$. Store at RT.

**6.** 0.0500 N $As_2O_3$, dissolve 2.4730 g dried $As_2O_3$ in 25 ml hot 1N NaOH in 1-L volumetric flask. Neutralize with 25 ml 1N $H_2SO_4$. Cool and dilute to vol with $H_2O$. Store at RT.

**Caution: $As_2O_3$ is extremely toxic--Avoid contact, handle in fume hood using gloves, mask, and goggles. Consult Material Safety Data Sheet for specific handling instructions.**

**7.** Phenol standard, dissolve 0.50 g phenol in 50 ml $H_2O$ and dilute to volume. Store at RT.

**Critical Control Point: The final diluted volume of the test fluid must be adjusted as described in 4.3.2.8.**

**8.** Test fluid (TF), dissolve 0.30 g $NaHCO_3$, 1.67 g $KBrO_3$, and 15.00 g KBr in $H_2O$ and qs to 1 L with $H_2O$. Store at RT. The TF volume must be adjusted by adding corrected volume of $H_2O$ to TF. It must take a volume of 21.3 ml to titrate 25 ml 0.050 N $As_2O_3$ in 10 ml "Acid Solution." A first time titration will require less than 21.3 ml TF. Adjust as described in the following example:

**Example: Assume the first time titration volume is 20.5 ml.**

(1,000 ml of TF)-(20.5 ml) = 979.5 ml

$\frac{(979.5)(\text{desired vol})}{(\text{actual vol})(20.5)}$ or $\frac{(979.5)(21.3)}{(\text{actual vol})(20.5)}$ = 1,017.2 ml

For corrected vol of $H_2O$: 1017.2 - 979.4 = 37.8 ml to be added to TF.

**Note:** TF in buret has to be put back into flask.

**3.4 Preparation of the sample**

**3.4.1 Receipt--**Reference current version of **TCSOP0001**.

**3.4.2 Preparation--**PPD tuberculin products are stored at 4°C in the walk-in refrigerator prior to testing. Before testing, allow sample vials and reagents to warm to room temperature.

**4. Performance of the test use Tuberculin (PPD) Log Sheet, Appendix 8.1**

**4.1 Protein--**Analyze the control pool and protein standard each time testing is performed. Analyze each in triplicate.

**4.1.1** Place 5.0 ml PPD, ½ Hg tablet, and 3.0 ml ± 0.1 ml $H_2SO_4$ into a Buchi digestion tube. Same for the standard and control.

**Caution: HgO is poisonous--Handle in fume hood, using gloves, mask, and goggles. Consult Material Safety Data Sheet for specific handling instructions.**

**Caution: Concentrated $H_2SO_4$ is corrosive--Handle in fume hood. Avoid contact with skin.**

**4.1.2** On digestion rack heater, digest for 30 min after all $H_2O$ is distilled and acid comes to true boil.

**4.1.3** Cool, add 3 ml $H_2O$ and cool again. Add 15 ml NaOH solution to sample and attach to distillation apparatus.

**4.1.4** Distill about 25 ml into 50-ml erlenmeyer flask containing 5 ml $H_3BO_3$ and 3 drops indicator.

**4.1.5** Titrate to endpoint color change of yellow to deep rose (pH 5.0) with HCl. Record result on log sheet.

**4.2 Phenol** (Analyze the control pool and phenol standard each time testing is performed. Analyze each in triplicate.)

**4.2.1** Add 5 ml PPD and 100 ml CS to 250-ml glass stoppered flask. Shake 2 min. Filter through fast filter paper.

**4.2.2** Transfer 50 ml filtrate to another flask. Add 1 drop methyl orange, stopper, and shake a few sec. Observe the color, when red, go to **4.2.3**.

**4.2.3** Titrate with 2 ml test fluid (TF), stopper, and shake a few sec and observe the color. When red, repeat **4.2.3**. When colorless, go to **4.2.4**.

**4.2.4** Shake 30 sec. Add 1 drop indicator, stopper, and shake a few sec and observe the color. When it does not turn to colorless within 10 sec, titrate with 1 ml TF, stopper, and repeat **4.2.4**. When colorless, go to **4.2.5**.

**4.2.5** Shake 1 min. Add 1 drop indicator, stopper, and shake a few sec. Observe the color. When red stays longer than 10 sec, titrate with 0.50-ml TF, stopper, and repeat **4.2.5**. When colorless, record total vol of TF as the endpoint of titration and use for calculation of percent phenol.

## 5. Interpretation of the test

**5.1 Protein** (Report average of triplicates.)

mg Protein/ml = (ml HCl)(N HCl)(1.4007)(6.25)/(5 ml PPD)
Satisfactory Protein Content: 1.0 mg/ml ± 0.1 mg/ml

**5.2 Phenol** (Report average of triplicates.)

Percent phenol = (vol of test fluid)(0.04)-(0.04)
Satisfactory Phenol Content: 0.50% ± 0.04%

### 5.3 Controls

Results for controls and standards must be within acceptable limits; otherwise repeat testing.

## 6. Reporting of test results

Validate and report results according to the current version of **TCSOP0001**.

## 7. References

**7.1** Code of Federal Regulations, Title 9, Section 113.409 Revised January 1992, page 629.

**7.2** Official Methods of Analysis of AOAC International, Arlington, Virginia, 16th Edition, Pat Cuniff, Editor (1995), Volume I, Chapter 12, page 7.

## 8. Summary of Changes

Version .01 was written to meet NVSL/CVB Quality Assurance requirements, to clarify practices in use in the NVSL/CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

Version .02 was written to clarify practices in use in the NVSL/CVB-L and to provide additional detail.

Version .03 was written to clarify practices in use in the NVSL/CVB-L and to provide additional detail. The following are the significant changes made from the superseded protocol:

**1**. Change in the digestion apparatus

Version .04 was written to clarify practices in use in the NVSL/CVB-L and to provide additional detail.

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## Appendix 8.1 TC Log Sheet

### Tuberculin (PPD) Log

Cases #: _____ Date: _____
Sample Code: _____ Analyst: _____
Serial #: _____ Reviewed by: _____
Comment: _____

**Protein** Micro-Kjedhal (5ml sample) [Vol. HCl] [N HCl] [1.4008] [10] [6.25] / (Vol. sample)

| Sample | | ____N / Vol. HCl | mg Protein/ml | Average of 3 |
|---|---|---|---|---|
| 1 _____ | a | | | _____mg/ml |
| | b | | | |
| | c | | | |
| 2 _____ | a | | | _____mg/ml |
| | b | | | |
| | c | | | |
| Std.: _____ | a | | | _____mg/ml |
| | b | | | |
| | c | | | |
| Control: _____ | a | | | _____mg/ml |
| | b | | | |
| | c | | | |

**Phenol** Titration: (5ml sample) (Vol. Test Fluid) (.04) - (.04)

| Sample | | Vol. Test Fluid | % Phenol | Average of 3 |
|---|---|---|---|---|
| 1 _____ | a | | | _____% |
| | b | | | |
| | c | | | |
| 2 _____ | a | | | _____% |
| | b | | | |
| | c | | | |
| Std.: _____ | a | | | _____% |
| | b | | | |
| | c | | | |
| Control: _____ | a | | | _____% |
| | b | | | |
| | c | | | |

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## 9. Quick reference

____ Product code _____

____ Submission paperwork correct

____ Received correct specimens and adequate amount

____ Protein standard, date of origin _____

____ Standard acid

____ Test fluid, date of origin _____

____ Control PPD, date of origin _____

____ Triplicate determinations conducted

____ Control phenol result within limits

____ Control protein result within limits

____ Protein standard result within limits

____ Phenol standard result within limits

____ Results entered into computer, date _____

____ Results validated, date _____

Analyst _____ Date _____